A D-Amino Acid Peptide Inhibitor of NF-κB Nuclear Localization Is Efficacious in Models of Inflammatory Disease

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The transcription factor NF-κB regulates many genes involved in proinflammatory and immune responses. The transport of NF-κB into the nucleus is essential for its biologic activity. We describe a novel, potent, and selective NF-κB inhibitor composed of a cell-permeable peptide carrying two nuclear localization sequences (NLS). This peptide blocks NF-κB nuclear localization, resulting in inhibition of cell surface protein expression, cytokine production, and T cell proliferation. The peptide is efficacious in vivo in a mouse septic shock model as well as a mouse model of inflammatory bowel disease, demonstrating that NF-κB nuclear import plays a role in these acute inflammatory disease models. *The Journal of Immunology,* **2000, 165: 1004–1012.**

Nuclear factor-κB is an inducible transcription factor comprised of homo- and heterodimers of p50, p65, p52, relB, and c-rel subunits whose effects on cells of the immune system have been widely studied (1–7). NF-κB is expressed as a cytosolic protein that translocates to the nucleus following cell activation, where it regulates the expression of a number of genes whose products are involved in inflammation, lymphocyte activation, and cell growth and differentiation. Therefore, inhibitors of NF-κB are important for the control of proteins involved in the amplification and perpetuation of chronic inflammatory disease (1). A number of NF-κB inhibitors have been described to date, including glucocorticoids (8), aspirin (9), gliotoxin (10), antioxidants such as pyrrolidine dithiocarbamate (PDTC) and acetylcysteine (11), and the cytokine IL-10 (12–13). However, glucocorticoids have endocrine and metabolic side effects when given systemically at high doses and for long periods of time. Antioxidants are relatively weak, short-lived, and affect kinase and phosphatase activity (14).

We report here a promising new approach to NF-κB inhibition using intracellularly targeted peptide inhibitors of nuclear translocation. They use short amino acid sequences termed nuclear localization sequences (NLSs)[2] (15–20), which mediate nuclear protein import. There are at least two categories of NLS: "simple," containing five to seven residues, or "bipartite," consisting of two sets of positively charged residues separated by 10–11 aa (15). While there is no universal consensus sequence among the known NLSs, they generally contain at least two basic amino acids which constitute the core of the NLS (15). The amino acid sequence of each NLS is specific and essential for its function; point mutations of critical basic residues destroy nuclear targeting activity (21, 22).

Facilitated nuclear import is a receptor-mediated process involving NLS-binding proteins, called karyopherins, which reside in the cytoplasm and at the nuclear pore (19). Several other cytoplasmic factors have also been shown to play a role in this process, including p10, Ran, and Hsc70 (23–25). Previous studies using the immunosuppressive drug deoxyspergualin, which has some structural similarities to the core NLS, have demonstrated that this agent inhibits nuclear transport of the transcription factor NF-κB perhaps by interfering with Hsc70-mediated nuclear import (26, 27). We have shown previously that distinct NLSs have varying affinities for the different forms of karyopherin α and that the different affinities play a major role in driving nuclear transport (28). Collectively, these studies suggested that 1) transcription factor nuclear transport plays a critical role in influencing cellular immune processes (29) and 2) inhibitors of nuclear translocation may be selective for NF-κB. We proposed that NLS peptides could be designed that would competitively inhibit the interaction between NF-κB and karyopherins and lead to an inhibition of the activation-induced transport of this transcription factor to the nucleus.

Other groups have shown that microinjection or treatment of cells with NLS peptides could inhibit nuclear transport in vitro (30, 31). The peptide we designed uses NLS sequences that alter the interaction of NF-κB with karyopherin α and block its transport into the nucleus. The NLS peptides are delivered into the cell by an amino acid sequence derived from fibroblast growth factor, which confers cell membrane permeability (32–34). Other examples of peptides that translocate through membranes include sequences from *Antennapedia* homeodomain, HIV Tat, and the HSV structural protein VP22 (33, 35).

Our peptide approach is different from the studies reported by Lin et al. (32) for several reasons. First, we used two NLSs, one on either end of the fibroblast growth factor translocation sequence. The cloning of karyopherin α and a recently published crystal structure shows that at least two NLSs can bind to a single molecule of karyopherin α (36). Therefore, we rationalized that delivery of multiple NLS peptides on a single molecule would have a much higher affinity for karyopherin α than a single NLS. Secondly, we synthesized the peptide in the D-amino acid form because it is known that D-form peptides are metabolized more slowly and are more resistant to proteolysis than their L-form counterparts. Finally, in contrast to the studies by Lin et al. (32), our

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Received for publication November 23, 1999. Accepted for publication May 8, 2000.

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[2] Abbreviations used in this paper: NLS, nuclear localization sequence; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease.

0022-1767/00/$02.00

studies focused on biological events downstream of NF-κB nuclear localization in cells of the immune system. This study demonstrates the efficacy of cell-permeable NLS peptide-induced inhibition of NF κB activation in modulating immune responses in vivo and in vitro.

Materials and Methods

Peptides

Peptides were synthesized on a Gilson multiple peptide synthesizer using F-moc (37) amino acids and purified by C_{18} reverse-phase HPLC. All peptides were analyzed by mass spectrometry and yielded the correct m.w.

Cells and reagents

The murine pre-B cell line 70Z/3 (American Type Culture Collection, Manassas, VA) was grown in RPMI 1640 supplemented with 10% heat-inactivated FBS and 5×10^{-5} M 2-ME. The murine monocyte/macrophage cell line RAW 264.7 (TIB71; American Type Culture Collection) was grown in DMEM containing 10% FBS. LPS was obtained from *Salmonella typhosa* 0901 (Difco, Detroit, MI). Anti-CD3 and anti-CD28 Abs were kind gifts of Hung Theh and Jim Allison, respectively (both of University of California, Berkeley, CA). Rabbit anti-hamster Ab was obtained from Jackson ImmunoResearch (West Grove, PA), and anti-κ Ig was obtained from Southern Biotechnology Associates (Birmingham, AL). Cytokine levels were measured using the appropriate Genzyme (Framingham, MA) ELISA kits. Human peripheral blood T, B, and monocytic cells were isolated by E-rosetting as described previously (38). For the IκB Western blot, we used Ab C-21 from Santa Cruz Biotechnology (Santa Cruz, CA). Dextran sodium sulfate (DSS) (m.w. = 40,000) was obtained from ICN Biomedical (Costa Mesa, CA).

EMSA

A total of 2×10^7 cells were washed once in PBS followed by preparation of nuclear extracts as described (14). The cells were lysed in buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM NaCl, 0.25% Nonidet P-40, pH 7.5) for 5 min at 4°C, followed by centrifugation at 4000 rpm for 5 min. The supernatant (cytosol) was removed, and the nuclei were extracted with buffer C (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.25% Nonidet P-40, pH 7.5). The nuclei were vortexed vigorously several times over 20 min, followed by centrifugation at 14,000 rpm for 5 min. The supernatant (nuclear extract) was removed and diluted 1:2 with buffer D (20 mM HEPES, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, pH7.5) and then frozen at −80°C until use. The ^{32}P-labeled probes used contained the sequence: NF-κB, 5′-AGTTGAGGGGACTT TCCCAGG-3′; AP-1, 5′-CGCTTGATGAGTCAGCCGGAA-3′; NF-AT, 5′-GGAGGAAAAACTGTTTCATACAGAAGG-3′; and Oct, 5′-TGTC GAATGCAAATCACTAGA-3′. A 6% polyacrylamide gel was used with 0.5× TBE as running buffer to resolve the complexes.

Measurement of cytokine secretion

RAW 264.7 cells (1×10^5/well) were preincubated for 2 h with BMS 205820 peptide before stimulation with LPS (0.5 ng/ml) and additional incubation for either 6 or 24 h. The culture supernatants were then analyzed for IL-10, IL-2, IL-6, or TNF-α levels by ELISA (Genzyme).

Mouse T cell purification and proliferation

To isolate splenic T cells, BALB/c spleens were teased apart and pressed through a wire mesh screen. The spleen cells were washed two times, and RBCs and other materials were removed using Lympholyte M (Cedarlane Laboratories, Westbury, NY). The spleen cells were enriched for T cells using a mouse T cell enrichment column (R&D Systems, Minneapolis, MN). The cells were then incubated at 37°C for 3 h with media (RPMI 1640 plus 10% FCS) plus or minus BMS 214572 at 0.5 or 2.0 μM. The cells were then centrifuged, resuspended in either media alone or containing the appropriate concentration of peptide, plated into 96-well plates, and stimulated as described in the figure legend. After 72 h, the cells were pulsed with [^{3}H]thymidine for 6 h and counts were determined.

Animal studies

For the sepsis model, BALB/c mice were administered a bolus injection of 200 μg LPS in PBS i.v., and mortality was measured after day 7. For the DSS study, Swiss-Webster mice were administered 5% DSS in drinking water from day 0 to day 6 and peptide on days 1–9. On day 10, the mice were sacrificed, and colons were removed and analyzed for disease. Sections of the entire colon were graded as to the severity of crypt injury and degree of inflammation. The crypt injury was scored as follows: grade 0, intact crypt; grade 1, loss of the basilar one-third of the crypt; grade 2, loss of the basilar two-thirds of the crypt; grade 3, loss of the entire crypt with the surface epithelium remaining intact; grade 4, loss of both the entire crypt and surface epithelium resulting in an erosion. The scoring severity of inflammation was as follows: grade 0, unremarkable; grade 1, minimal; grade 2, mild; grade 3, moderate; grade 4, severe. Both scores also included a measure of the extent (percentage) of involvement as follows: grade 1, 0–25%; grade 2, 26–50%; grade 3, 51–75%; grade 4, 76–100%. The final score is the product of either the inflammation or injury grade by the grade for extent of involvement.

Table I. *Sequences of NLS Peptides*

BMS-205820	PKKKRKVAAVALLPAVLLALLAPKKKRKV
BMS-214572	AKRVKLAAVALLPAVLLALLAAKRVKL
SV40	GGGPKKKRKV
c *myc*	AKRVKL
JBC	AAVALLPAVLLALLAPVQRKRQKLMP
MEM	AAVALLPAVLLALLA

The membrane-translocating hydrophobic sequence derived from the predicted signal peptide sequence of fibroblast growth factor is bold. The NLS derived from either SV40 large T antigen or c-*myc* are underlined. The peptides without the membrane translocation sequence were used as comparative controls. The peptide containing a single NLS derived from NF-κB is indicated as JBC.

Results

BMS 214572 and BMS 205820 inhibit the nuclear localization of NF-κB

We synthesized a D-amino acid peptide (referred to as BMS 214572) containing a cell membrane-translocating sequence flanked by two nuclear localization sequences derived from c-*myc* (16, 39) (Table I). We show elsewhere using confocal microscopy that BMS 214572 is readily taken up by cells within 30 min and accumulates in the cytoplasm (Nadler et al., manuscript in preparation). In all of the experiments described below, each peptide was used at concentrations that were nontoxic, in the time frame of the experiment, based on trypan blue exclusion (data not shown). In addition, we have also synthesized another peptide, BMS 205820, that contains the NLS from SV40 large T Ag (16, 40). To evaluate the effect of the peptides on NF-κB nuclear translocation, we performed EMSA using nuclear extracts from 70Z/3 cells. NF-κB activation in these cells has been well studied, making this cell line an ideal candidate for these studies. As shown in Fig. 1*A*, both BMS 205820 and BMS 214572 potently inhibited LPS-induced NF-κB translocation in the high nanomolar range. The two peptides also inhibited PMA-induced NF-κB translocation; however, the concentration of peptides required for inhibition was several-fold higher. The inhibition was selective for NF-κB, as Oct-1 DNA binding from the same extracts was not affected. The step immediately preceding nuclear import is the degradation of IκB (subsequent to phosphorylation) to expose the nuclear localization sequence on NF-κB (1). As seen in Fig. 1*B*, BMS 205820 at a concentration that was shown to inhibit NF-κB translocation did not affect the degradation of IκB, indicating that the peptide likely inhibits the nuclear import of NF-κB and not an upstream signaling pathway.

To assess the effects of the peptides on other cell types, we tested the ability of BMS 205820 to inhibit NF-κB in normal human PBL as well. As shown in Fig. 2, NF-κB nuclear localization in T cells was inhibited only by 2 μM peptide, whereas the B plus monocyte cell population was sensitive to inhibition by both concentrations of the peptide. There was no significant effect on the nuclear levels of octamer binding protein. Together with the data showing differential effects on PMA vs LPS nuclear transport,

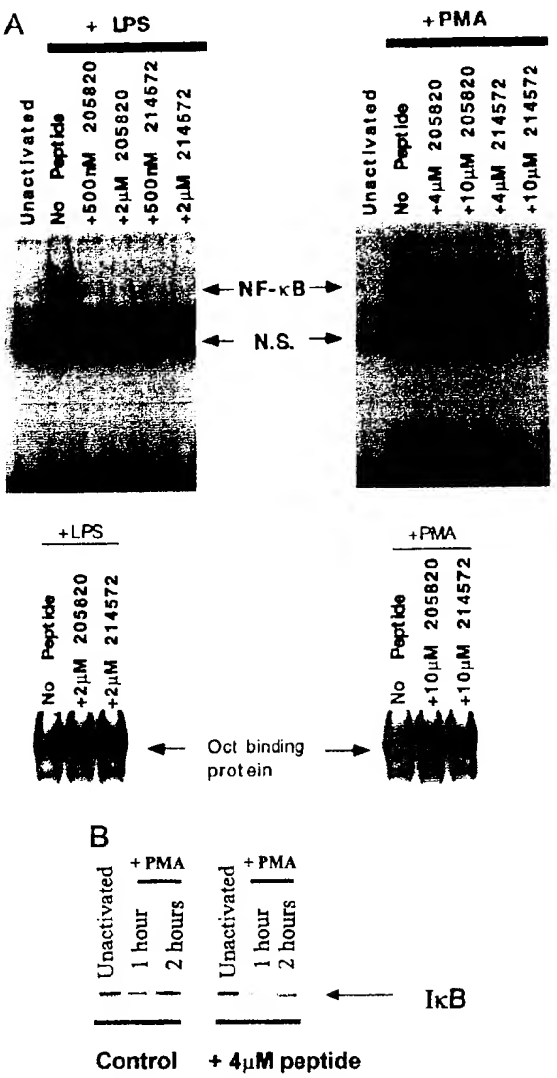


FIGURE 1. BMS 214572 and BMS 205820 inhibit NF-κB nuclear translocation in 70Z/3 cells. *A*, Mouse 70Z/3 pre-B cells were pretreated with the indicated concentrations of BMS peptides for 3 h before activation with 10 ng/ml LPS or 5 ng/ml PMA for 1 h. The cells were lysed and nuclear extracts were prepared and analyzed by EMSA for the presence of NF-κB or Oct-1 binding proteins. *B*, 70Z/3 cells were treated with 4.0 μM of BMS 205820 before activation with 5 ng/ml of PMA for the indicated times. Cytoplasmic extracts were prepared and equal protein levels were run in each lane on a SDS-polyacrylamide gel and Western blotted with an anti-IκB Ab.

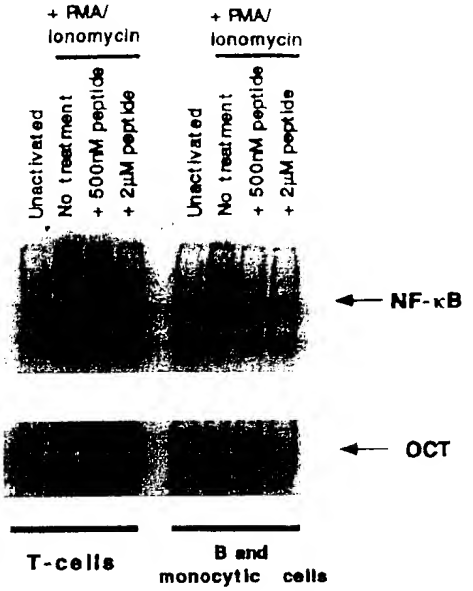


FIGURE 2. BMS 205820 inhibits NF-κB nuclear localization in human T, B, and monocytic cells. Human PBMC were isolated, separated into T cell and B cell plus monocyte subsets by E-rosetting techniques, and pretreated for 3 h with either 500 nM or 2 μM BMS 205820. The cells were then activated for 1 h with 10 ng/ml PMA plus 1 μM ionomycin. Nuclear extracts were prepared and analyzed by EMSA for presence of NF-κB or Oct-1.

these data suggest there may be different nuclear transport pathways in different cell types. The different concentrations of peptide required for inhibition of NF-κB in different cell types and methods of stimulation may also be due to different levels of karyopherins. In fact, we have shown that there is a differential expression of karyopherins in different cell types and that stimulation of cells with PMA causes a rapid increase in the levels of karyopherins compared with LPS stimulation (28).

BMS 205820 inhibits expression of LPS-induced κ Ig and other cell-surface Ags

To determine the effect of BMS 205820 on NF-κB-regulated immune responses in vitro, we used 70Z/3 cells as a model of B cell differentiation (41). These cells can be activated to express surface IgM, indicative of conversion to a more mature B cell phenotype. This increase in surface IgM expression is due to induction of κ light chain transcription, which is regulated in large part by NF-κB. We stimulated 70Z/3 cells overnight with 500 ng/ml LPS, under which conditions ~95% of cells become κ light chain positive (data not shown). Fig. 3*A* shows that both the L-amino acid form and the D-amino acid form of BMS 205820 are equally effective at inhibiting LPS-induced κ expression. As predicted based on its ability to inhibit NF-κB, BMS 214572 was also very effective at inhibiting surface κ expression. In contrast, the single NLS L-amino acid JBC peptide was significantly less inhibitory, as were the SV40 and translocation peptide controls.

70Z/3 cells can also be induced by IFN-γ to express surface κ Ig. IFN-γ is known to activate the Jak/STAT transcription factor pathway, which is NF-κB independent (42). Interestingly, we found that IFN-γ-induced κ expression was not affected by BMS 205820 (Fig. 3*B*), suggesting that the peptide inhibition is indeed selective for the NF-κB-mediated signaling pathway.

Besides κ Ig, other genes with putative NF-κB binding sites include cell-surface molecules involved in immune function such as CD40, class I and class II MHC, and cell adhesion molecule CD54 (1, 2). We examined the effect of the peptide on surface levels of these molecules and found that BMS 214572 was effective at inhibiting LPS-induced but not IFN-γ-induced surface expression of CD40, class I MHC, and CD54, whereas class II MHC was not affected (data not shown).

To determine whether the peptide could inhibit an ongoing immune response, we treated 70Z/3 cells with the peptide at various times after LPS activation. BMS 205820 was able to maximally inhibit κ expression only when added before or at the same time as the LPS stimulus (Fig. 3*C*); the inhibitory effect of the peptide decreased when added after NF-κB activation. These data are consistent with the hypothesis that the peptide is inhibiting translocation of NF-κB to the nucleus rather than interfering with later

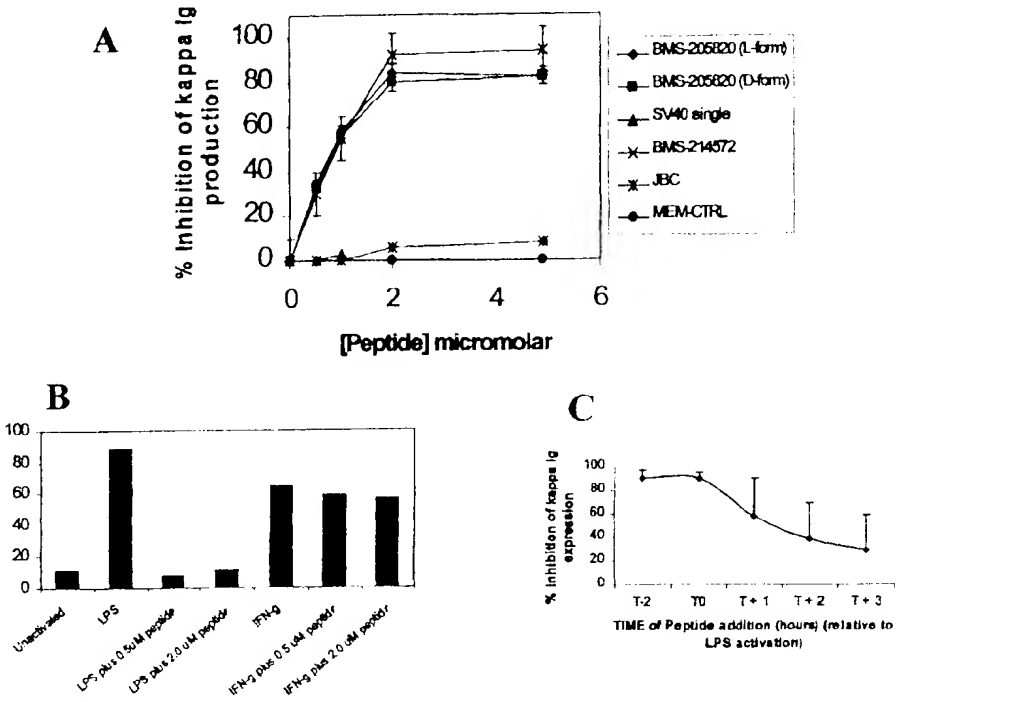


FIGURE 3. BMS 214572 and BMS 205820 peptides inhibit LPS- but not IFN-γ-induced κ Ig light chain expression on 70Z/3 cells, and inhibition decreases when added after LPS activation. *A*, Mouse 70Z/3 pre-B cells were pretreated with the indicated concentrations of BMS 205820, BMS 214572, or various control peptides for 3 h followed by overnight activation with 500 ng/ml LPS. Cells were stained with a FITC-anti-κ Ig (Southern Biotechnology Associates) and analyzed by FACS. The SV40 single peptide was not used above 1 μM due to cytotoxicity. Data are expressed as mean ± SD. *B*, 70Z/3 cells were pretreated with the indicated concentrations of BMS 205820 peptide for 3 h before overnight stimulation with either 500 ng/ml LPS or 50 U/ml IFN-γ. Cells were stained and analyzed as above. *C*, 70Z/3 cells were treated with 2 μM BMS 205820 at the indicated time relative to LPS activation. The cells were then activated overnight with 500 ng/ml LPS and stained and analyzed as above. Data are expressed as mean ± SD.

events such as DNA binding or cellular processes in general. In fact, we have shown that there was no effect on NF-κB binding to DNA at concentrations of peptide up to 1 μM (data not shown).

BMS 205820 and BMS 214572 inhibit proinflammatory cytokine production

Production of proinflammatory cytokines by monocytes is known to be regulated by transcription factors such as NF-κB. TNF-α, in particular, appears to be involved in disease states such as rheumatoid arthritis, inflammatory bowel disease (IBD), and septic shock (1, 43). Therefore, we examined the effect of BMS 205820 on TNF-α and IL-6 cytokine production induced by LPS activation of RAW 264.7 cells. As shown in Fig. 4, BMS 205820 at 5 μM nearly completely inhibited both TNF-α and IL-6 production in response to LPS. BMS 214572 had a similar ability to suppress TNF-α production. In contrast, neither the c-*myc* nor SV40 single NLS peptide had much effect in these assays.

Effect of BMS 214572 on T cell proliferation

To assess the peptide effect on other cells of the immune system, mouse splenic T cells were examined in a proliferation assay. As shown in Fig. 5*A*, when T cells were stimulated with anti-CD3 plus anti-CD28, pretreatment with increasing amounts of peptide resulted in an augmentation of proliferation. However, when cells were stimulated instead with PMA plus anti-CD28, the peptide effect was reversed and T cell proliferation was inhibited. Both effects were most dramatic when low concentrations of the stimuli were used (data not shown). There was no toxicity observed in the time frame of the experiment. One explanation for these divergent

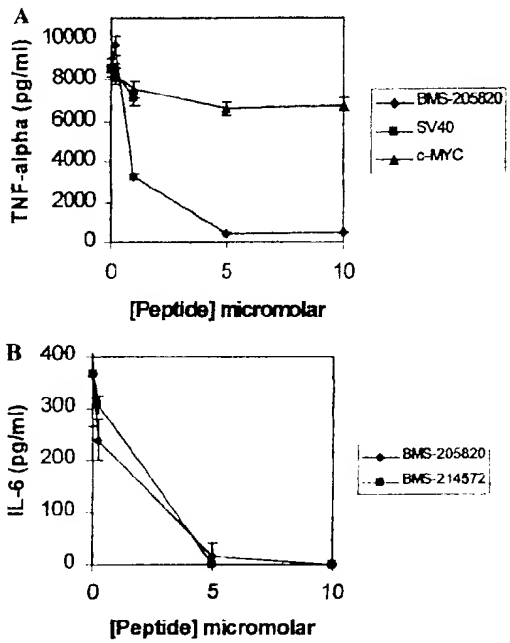


FIGURE 4. BMS 214572 and BMS 205820 inhibits LPS-induced IL-6 and TNF-α production in RAW 264.7 cells. Mouse RAW 264.7 cells were pretreated with the indicated concentrations of peptides for 3 h followed by activation with 10 ng/ml LPS for 6 h. The cell supernatants were harvested and (*A*) TNF-α and (*B*) IL-6 cytokine levels were measured by ELISA. Data are expressed as mean ± SD.

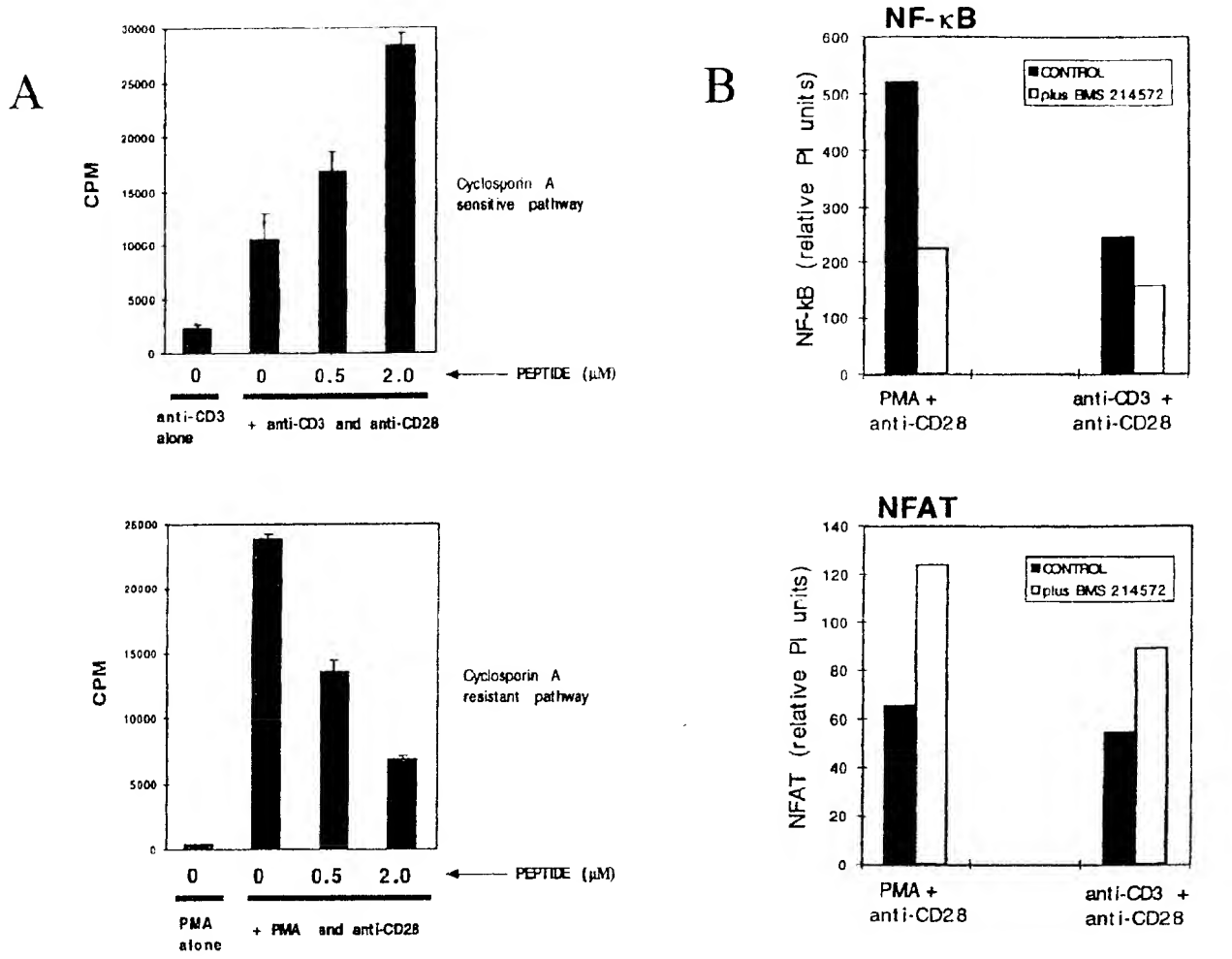


FIGURE 5. BMS 214572 affects mouse splenic T cell proliferation and transcription factor nuclear localization. *A*, Splenic T cells from BALB/c mice were isolated and pretreated with either 0.5 or 2.0 μM peptide for 2 h followed by activation with either anti-CD3 alone (1.25 μg/ml), PMA alone (1 ng/ml), anti-CD3 plus anti-CD28 (1.25 μg/ml each), or anti-CD28 plus PMA (0.5 mg/ml and 1 ng/ml, respectively). Proliferation was determined after 72 h by pulsing for 8 h with [^{3}H]thymidine. Data are expressed as mean ± SD. *B*, EMSA analysis of mouse T cell nuclear extracts. T cells were pretreated with 2 μM BMS 214572, activated as described above, and nuclear extracts were prepared. An EMSA analysis was performed and the gels analyzed and quantified by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA). Unactivated cells were assigned a phospholmager (PI) unit of 0.

effects is that the CD3 plus CD28 activation pathway is calcium dependent and partially cyclosporin A sensitive and therefore signals predominantly through a different transcription factor such as NF-AT (44, 45). BMS 214572, by inhibiting NF-κB signaling, may act to indirectly enhance NF-AT-mediated T cell activation. In contrast, PMA plus anti-CD28-induced proliferation, which is cyclosporin A resistant, may be more NF-κB dependent. Therefore, peptide inhibition of T cell activation would be more evident under these conditions.

We addressed this possibility by testing the nuclear extracts from these treated cells in EMSA to determine whether inhibition of NF-κB activation correlated with inhibition of proliferation. We observed that stimulation of T cells with PMA plus anti-CD28 resulted in significant NF-κB activation, and to a greater extent than anti-CD3 plus anti-CD28 activation, as shown in Fig. 5*B*. When cells were pretreated with BMS 214572 under these conditions, NF-κB activation was inhibited. Although stimulation of T cells with anti-CD3 plus anti-CD28 resulted in a much lower level of NF-κB activation compared with PMA plus anti-CD28, pretreatment with peptide to a limited extent inhibited activation by this stimulus as well. Both methods of stimulation resulted in similar levels of NF-AT activation. Interestingly, we found that there was an increase in binding of nuclear extract proteins to an NF-AT DNA probe when cells were treated with peptide.

These data are consistent with the hypothesis that PMA plus CD28 stimulation signals primarily through the NF-κB pathway, unlike CD3 plus CD28 stimulation, which signals mainly through a non-NF-κB pathway. In addition, we examined Oct-1 activation and found little effect of the peptide on this transcription factor, further demonstrating the specificity of the peptide for NF-κB (data not shown). Possible explanations for the differential effects on proliferation induced by the two treatments are discussed below.

BMS 205820 inhibits in vivo inflammatory responses

Because of the intriguing in vitro results reported above, we were interested in the effect of the peptide on in vivo models of disease. As shown in Table II, BMS 205820 was able to significantly protect mice from death in a murine model of septic shock (46). Because the biologic consequences of sepsis are widely thought to

Table II. *Effect of BMS 205820 in an LPS-induced lethal mouse model of septic shock*[a]

Fraction of Mice Surviving 7 Days Post LPS	
LPS + PBS	LPS + BMS 205820
2/10*	10/10

[a] BALB/c mice were administered 200 μg of LPS plus or minus 5 mg/kg BMS 205820. The peptide was administered i.v. immediately after LPS treatment and 24 h later. Mortality was measured on day 7. The survival rate of the peptide-treated group is higher than the PBS alone (*, $p < 0.001$ using a log-rank test).

involve proinflammatory cytokines such as TNF-α, we also treated mice with a nonlethal dose of LPS and measured cytokine levels. TNF-α levels in these mice were shown to be suppressed by peptide treatment. IL-6 levels were only slightly affected, and IL-10 levels were significantly enhanced (Fig. 6). Similar to the peptide-induced increase on anti-CD3 plus anti-CD28-mediated T cell proliferation, it is possible that NF-κB acts in a negative fashion to limit IL-10 production. The efficacy of the peptide inhibitor in the sepsis model suggests it may play a role in treatment of this disease.

We also tested the peptide in a second model of inflammation, the well-established mouse DSS-induced IBD model. The mouse DSS model has been shown to mimic many of the pathologies seen in ulcerative patients and some of which are seen in Crohns disease as well (47). Disease induced by DSS is characterized by epithelial damage, transmural inflammatory infiltrate, and lymphoid hyperplasia. As seen in Fig. 7 (*top*), there were dramatic changes in the histopathology of the colons of DSS-treated mice before and after per os or i.v. treatment with peptide. These changes are quantified in Fig. 7 (*bottom*), which illustrates a peptide-mediated decrease in both the inflammation and injury scores of the diseased colon. These data demonstrate that BMS 205820 is efficacious in this mouse model of IBD.

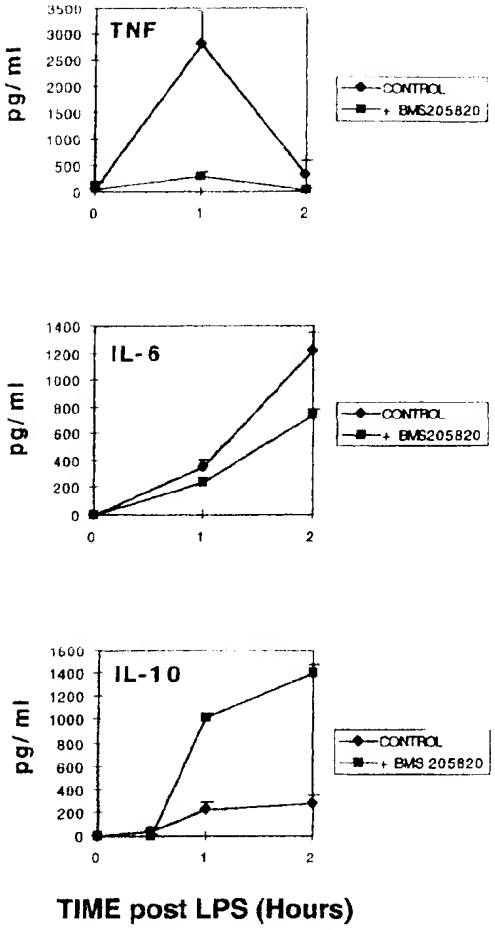


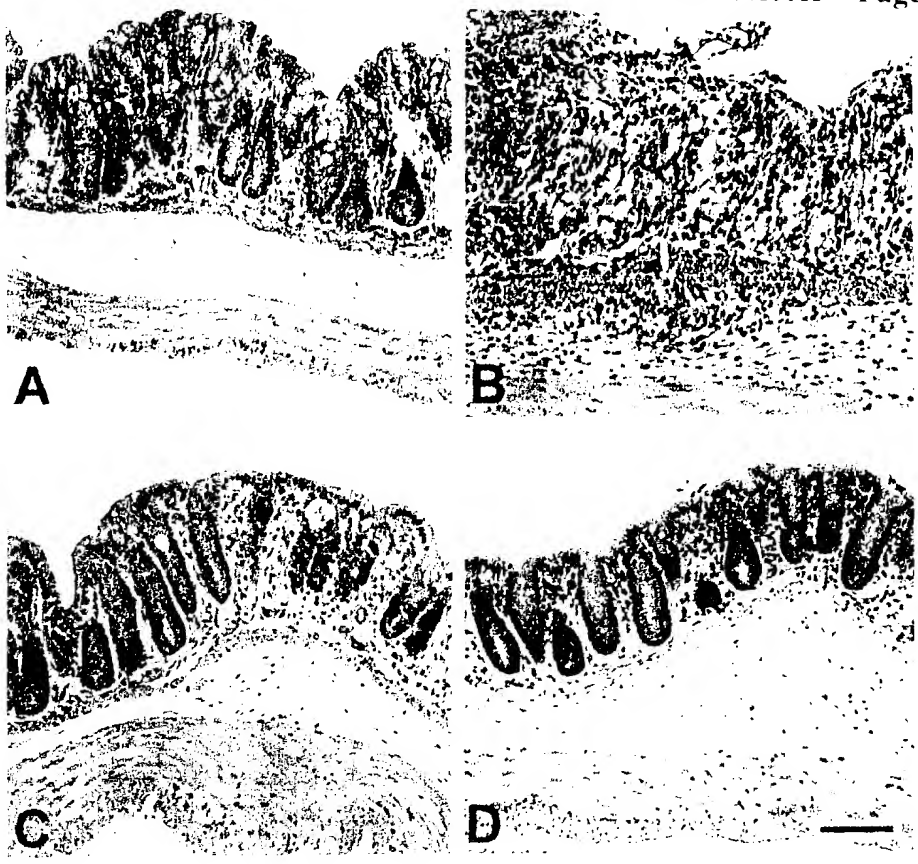
FIGURE 6. BMS 205820 modulates cytokine levels in vivo. BALB/c mice were administered 1 μg LPS i.v. and a single dose of peptide (5 mg/kg) was administered immediately thereafter. Blood samples were taken at the indicated times and cytokine levels were measured by ELISA. Data are expressed as mean ± SD. *, $p < 0.0001$ between control and peptide treated samples using a one-way ANOVA.

Discussion

This is the first report describing the in vitro and in vivo effects of an intracellular targeted D-amino acid peptide inhibitor of NF-κB. Most of the previously described inhibitors of NF-κB have been shown to act on the early stages of NF-κB activation (8–13). The peptide we designed acts at the more downstream event of nuclear translocation; hence, this peptide inhibitor represents a novel means of modulating transcription factor activity.

The mechanism of action of the peptides is established; we have shown that BMS 214572 and BMS 205820 block the nuclear localization of NF-κB. There was also a dose response relationship between inhibition of NF-κB nuclear translocation and the downstream effect on κ light chain production (Fig. 1, *left* vs Fig. 3*B*). This inhibition was selective for NF-κB, as translocation of other nuclear transcription factors such as Oct-1 and NF-AT was not significantly inhibited. In addition, there was no effect of the peptides on IFN-γ/STAT-mediated processes. While our data suggest that these peptides are specific for inhibition of NF-κB, we cannot rule out the possibility that the localization of other proteins is affected as well. We predicted the effect would be most specific for NF-κB due to its proposed lower affinity interaction (compared with other transcription factors) for karyopherin α. Torgerson et al. (34) recently reported that an NLS peptide based on the NF-κB p50 sequence attached to the fibroblast growth factor translocation sequence inhibited the nuclear translocation of several transcription factors. Those results could be attributed to the different peptide sequences used in their studies and suggest that there are multiple pathways and various ways to modulate the import process. Indeed, we found that the SN50 peptide reported by Torgerson et al. (34), which has the identical sequence to the JBC peptide reported herein, was ~35-fold less effective at inhibiting κ Ig expression compared with BMS 205820. These differences in potency may contribute to the differences in selectivity seen with these two peptides. In addition, SN50 may bind to different armadillo motifs on karyopherin relative to BMS 205820, resulting in differences in selectivity as well.

The inhibition by BMS peptides does not appear to be due to a toxic effect, because cell viability and global protein levels, both cytoplasmic and nuclear, were unaffected (data not shown). Not all NF-κB-regulated biological functions were affected by the peptides, and some were affected to varying degrees, possibly because redundancy within the cell allows other transcription factors to compensate for the loss or inhibition of NF-κB. One example is the lack of inhibition of LPS-induced class II expression that is reported to be regulated by NF-κB. In addition, the peptide-mediated inhibition was overcome when higher doses of LPS were used to stimulate cells, suggesting that NF-κB-mediated immune responses may require a threshold level of NF-κB to activate transcription (data not shown). Alternatively, higher concentrations of



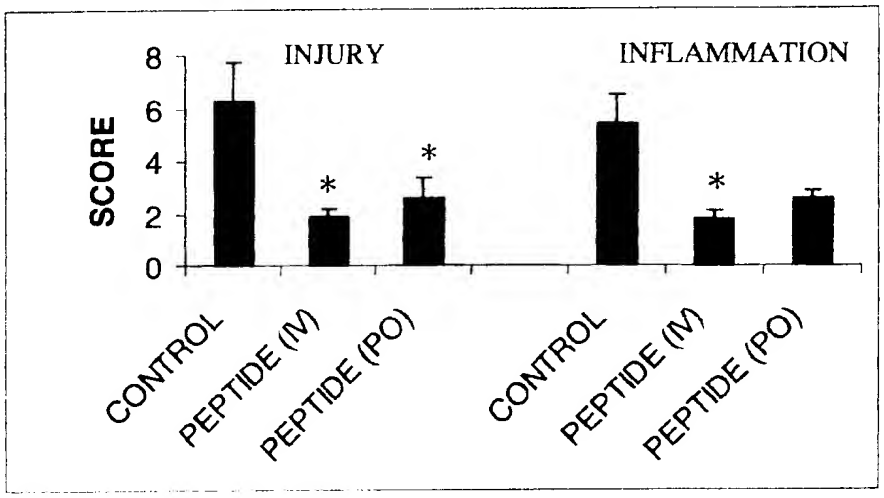


FIGURE 7. BMS 205820 is efficacious in a mouse DSS-induced IBD model. *A*, Colon from an untreated Swiss-Webster mouse (i.e., no DSS). There are no histomorphologic changes. *B*, Colon from a mouse receiving 5% DSS and treated with PBS. There is a broad-based ulcer characterized by disruption of the mucosal epithelium and loss of glandular crypts. The lamina propria contains incipient fibrous connective admixed with moderate numbers of lymphocytes, macrophages, and neutrophils. *C*, Colon from a mouse receiving 5% DSS and oral administration of BMS 205820 at 5 mg/kg. There is mild glandular loss with minimal inflammation in the lamina propria. *D*, Colon from a mouse receiving 5% DSS and i.v. BMS 205820 at 0.3 mg/kg. The glandular epithelium is intact and few inflammatory cells are present in the lamina propria. All sections stained with hematoxylin and eosin with similar magnification; bar in *D*, 75 μm. *Bottom*, Injury and inflammation scores from DSS with or without peptide-treated mice. The scores were derived as described in *Materials and Methods*. Data expressed as mean ± SD. *, $p \leq 0.01$, difference between control and peptide-treated samples using a multiple ANOVA analysis.

LPS may trigger other signaling pathways that are able to mediate the same biological events. These possibilities are being examined.

The peptide inhibitor of NF-κB that we describe in this report has yielded some surprising results with respect to the modulation of immune responses, compared with other inhibitors of NF-κB. We have begun to determine a molecular basis for the differential effects of the peptides on T cell proliferation. The effects do not appear to be due to changes in IL-2 or IL-4 because the levels of these cytokines were not significantly altered by peptide treatment (data not shown); effects on the synthesis of other cytokines are presently being investigated. Although IL-2 has been reported to be regulated by NF-κB, we have found that peptide inhibition of NF-κB did not affect IL-2 levels in mouse cells. This suggests that other transcription factors such as NF-AT may be sufficient for driving transcription of the IL-2 gene. With respect to the stimulation of proliferation by the peptide upon CD3 plus CD28 activation, it is possible that NF-κB may be acting as a negative regulator of transcription. Inhibition of NF-κB by the peptide may actually enhance transcription of certain genes. In fact, Casolaro et al. (48) have shown that NF-κB inhibits NF-AT-driven transcription in particular T cell subsets. We also found that the peptide enhanced NF-AT DNA binding, although at this point we do not

have an explanation for these results. Additional studies on the effects of the peptides on T cells should further our understanding of the role of NF-κB on T cell responses.

The efficacy exhibited by the NLS peptides in two important established in vivo models of inflammatory disease is encouraging. Not only were animals protected from septic shock death, but TNF-α, which is one of the players implicated in septic shock, was significantly decreased whereas IL-10 levels were dramatically enhanced. Because IL-10 is antiinflammatory, its presence serves to limit the production of proinflammatory cytokines. Interestingly, compounds such as SR31747A, glucocorticoids, and cAMP enhancing agents (49) also cause inhibition of TNF-α and concomitant increases in IL-10 in response to LPS (50). Some of these compounds have also been shown to inhibit NF-κB. The effects on IL-10 may either be transcriptional or a secondary effect of decreasing TNF-α levels. Reports describing high levels of activated NF-κB in the inflamed bowels of Crohn's disease patients as well as enhanced expression of NF-κB-regulated proinflammatory cytokines such as IL-1 and TNF-α implicate the transcription factor as a key player in IBD (51, 52). Because of the fundamental roles of IL-1 and TNF-α in the inflammatory response, inhibition of these cytokines would be expected to provide effective treatment of IBD. In fact, local administration of antisense oligonucleotides targeted against p65 NF-κB abrogated established intestinal inflammation in mice, providing direct evidence for the importance of NF-κB in IBD (53). Remarkably, the BMS 205820 peptide was efficacious when administered i.v. as well as when administered orally. Because of the resistance of the D-amino acid peptide to proteolysis, we believe that the peptide may be acting topically at the site of inflammation in the colon. The DSS model is very stringent, and, in fact, standard therapies such as steroids are not effective in this model although they are potent antiinflammatory agents in humans. That BMS 205820 is efficacious in this model suggests it may be quite potent and indicates its potential utility in treating inflammatory disease patients. In order for this peptide to be used clinically it must first be shown to be safe in animals. A 5-day i.v. dosing study in rats at doses up to 10 mg/kg showed no toxicities or histopathologic changes (data not shown). In addition, a 2-wk oral dosing study in mice up to 20 mg/kg showed no overt signs of toxicity or histopathologic changes, indicating that this compound is safe in animals. In both of these toxicology studies, a detailed histopathologic analysis of all organs and leukocytes was performed.

Our work using cell-permeable nuclear-targeted D-amino acid NLS peptides presents a useful tool for studying gene transcription and downstream intracellular processes specific to a single transcription factor. We have shown that these peptides inhibit a variety of NF-κB-dependent immune responses in several different cell types, both in vitro and in vivo. Based on these results, we propose that these peptides have potential as novel immunomodulatory therapeutic agents. Future studies will determine whether modulating gene expression at the level of nuclear transport is applicable to transcription factors other than NF-κB (54).

Acknowledgments

We thank Dr. Alejandro Aruffo for his support of this project and review of the manuscript. We also thank Dr. Ira Mellman for helpful advice.

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